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TECHNICAL NOTE

Demonstration of extracellular immunoproteins in formalin-fixed renal biopsy specimens

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Immunohistochemical techniques have been used for many years to establish the pathogenesis of renal disease. Deposits of immunoglobulin and complement are present in granules in glomerular capillary walls and mesangium in immune-complex disorders, whereas linear deposits of immunoglobulin and complement are found within the capillary walls in disorders due to antibodies directed against the glomerular basement membrane [1]. The diagnosis and classification of renal disease is often based on the detection of specific immunoglobulin classes and their site of deposition [2]. Either immunofluorescent or immunoperoxidase methods can be applied to renal tissue that has been frozen at the time of biopsy, and the two methods give similar results [3–5]. In almost every case, these methods are unable to demonstrate extracellular deposits of immunoglobulin and complement if the tissue has been fixed in formalin. Following the reports of Huang, Minnasian, and More [6] and of Curran and Gregory [7], we have investigated the use of an immunoperoxidase technique on formalin-fixed wax-embedded needle-biopsy specimens of the kidney after prior treatment with trypsin. We have used the unlabeled antibody, peroxidase-antiperoxidase (PAP) method of Sternberger [8] and have shown that controlled treatment with trypsin prior to the PAP method enables extracellular deposits of both immunoglobulin and complement to be demonstrated. The results are compared with those obtained from standard immunofluorescent methods performed on renal tissue frozen at the time of biopsy.

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Methods

The trypsin-PAP technique was performed on percutaneous needle biopsy specimens on kidneys fixed in 10% neutral buffered formal saline for at least 12 hours, processed to wax, and stored at room temperature, in some instances for as long as 6 years. We examined cases of membranous glomerulonephritis (five cases), membranoproliferative glomerulonephritis type-I (three cases) and type-II dense deposit disease (one case), crescentic glomerulonephritis with granular deposits (one case) and with linear deposits (three cases), as well as 15 examples of diffuse proliferative glomerulonephritis. Three-micron sections were floated out on water at 50° C, mounted on clean glass slides, and dried at 60° C for 30 to 60 min. Endogenous peroxidase was inhibited by methanol and hydrogen peroxide, and sections were incubated at 37° C in 0.1% trypsin (Code T8128, Sigma) dissolved in distilled water containing 0.1% calcium chloride and adjusted to a pH of 7.8 with 0.1 N sodium hydroxide [6]. We prepared 0.05% solutions by dilution with glass-distilled water. Trypsin solutions were used within 1 hour of preparation. The PAP technique [9] was carried out (Table 1). The effect of varying the incubation time from 20 to 60 min was examined with tissue known to contain extracellular immunoglobulin and complement components. Antisera raised in rabbits to the heavy chains of IgG, IgA, IgM, and IgD and to complement components Clq, C4, and C3 were used (Behringwerke AG). The optimal dilution for each antiserum was determined by titration, and the lowest concentrations that produced positive staining in sections known to contain extracellular serum protein were used. This was always between 1/400 and 1/4000. The concentrations of both the antiglobulin bridge (swine antirabbit IgG, Nordic Immunological Reagents) and the soluble complexes of PAP (peroxidase-rabbit anti-

peroxidase; Dakopatts AG) were determined by checker-board titration on positive-control tissue sections, as before. Peroxidase was demonstrated by the technique of Graham and Karnovsky [10] with hydrogen peroxide and diaminobenzidine, which is a potential carcinogen and should be handled with care. Immunologic controls consisted of the blocking (inhibition) technique. Excess goat antiserum, raised against each of the human proteins, was inserted between steps 7 and 8 of the technique (see Table 1). This produced a significant diminution in intensity of positive staining [11]. The specificity of all antisera was confirmed by immunoelectrophoresis and immunodiffusion methods.

The immunofluorescent technique was carried out on percutaneous needle-biopsy specimens that had been snap frozen and stored in liquid nitrogen. Fresh frozen sections were cut at 7 μm in a cryostat, mounted on alcohol-cleaned glass slides, and air dried for 5 min, before being placed in a moist chamber at room temperature (18 to 20° C). The immunofluorescent technique was then carried out with the direct method (Table 2) for demonstrating heavy chains IgG, IgA, IgM, and complement C3 by using swine or goat antihuman sera labeled with fluorescein isothiocyanate (FITC; Nordic Immunological Reagents Ltd.). The indirect method (Table 3) was used to demonstrate the heavy chain of IgD and for complement components C4 and Clq with sera raised in rabbits against the human proteins (Behringwerke AG), followed by goat antirabbit serum labeled with FITC (Nordic Immunological Reagents Ltd.). The appropriate dilutions for all antisera were determined by titration with control-positive sections, and the lowest titer that gave positive demonstration of protein was used. All sera were tested by immunodiffusion and immunoelectrophoresis to confirm specificity. Control sections were used in all instances to confirm positive reactions. These consisted of the blocking or inhibition test as a control for the direct immunofluorescent technique with antisera raised in rabbits. The indirect technique was controlled by ommission of the primary antiserum, rabbit anti-IgD, Clq, or C4, respectively.

Table 1. Trypsin-immunoperoxidase (PAP) technique

1) De-wax and place sections in alcohol
2) Inhibit endogenous peroxidase, 0.5% hydrogen peroxide, in methanol for 10 min
3) Wash in glass distilled water for 10 min
4) Incubate in 0.1% or 0.05% trypsin solution at 37° C for 20 to 60 min
5) Wash in cold glass-distilled water for 10 min
6) Transfer to a moist chamber, and wash twice in 0.5 M Tris/HCl buffer (pH, 7.6) diluted 1/10 with saline (TBS) for 10 min each
7) Treat with normal swine serum diluted 1/5 in TBS for 10 min
8) Drain off and replace with rabbit antiserum raised against human protein, diluted in TBS for 30 min
9) Wash in TBS, 3 times, for 10 min
10) Apply swine anti-rabbit IgG diluted 1/80 in TBS for 30 min
11) Wash in TBS, 3 times for 10 min
12) Apply PAP diluted 1/200 in TBS for 30 min
13) Wash in TBS, 3 times for 10 min
14) Demonstrate peroxidase with 5 mg 3,3'-diaminobenzidine tetra-HCl dissolved in 10 ml 0.2 M Tris/HCl buffer (pH, 7.6)—immediately before use add 0.1 ml 1% H_2O_2—for 10 min [10]
15) Wash in running tap water
16) Counterstain with Mayers hemalum, blue, dehydrate, clear, and mount in DPX

Table 2. Immunofluorescence technique—direct method

1) Wash in 0.01 M phosphate-buffered saline (PBS) (ph, 7.3) 3 times for 10 min
2) Apply FITC-labeled antiserum to human serum proteins diluted 1/20 in 0.9% saline for 30 min
3) Wash 3 times in PBS for 10 min
4) Mount in 90% glycerol in PBS—store at 4° C overnight prior to examination under UV light

Table 3. Immunofluorescence technique—indirect method

1) Wash in PBS, 3 times for 10 min
2) Apply unlabeled rabbit antiserum to human protein, diluted 1/20 in 0.9% saline for 30 min
3) Wash in PBS, 3 times for 10 min
4) Apply FITC-labeled goat antirabbit serum diluted 1/20 in 0.9% saline
5) Wash twice in PBS for 10 min
6) Mount in 90% glycerol in PBS, store at 4° C overnight prior to examination under UV light

Results and Discussion

Neither immunoglobulin nor complement could be detected in paraffin sections of renal biopsy specimens examined by standard immunoperoxidase methods (Fig. 1a). By using controlled trypsin digestion prior to the immunoperoxidase (PAP) technique, however, we demonstrated proteins in glomeruli (Fig. 1b), tubular lumina, tubular cell cytoplasm, or peritubular capillaries. Methanol and hydrogen peroxide effectively inhibited endogenous peroxidase. Neither collagen, elastic, or lipofuscin caused confusion in interpretation, and immunologic controls confirmed the specificity of positive reactions. Clq complement and the heavy chains of immunoglobulins IgG, IgA, IgM, and IgD were detected in the glomeruli of fixed tissue following incubation at 37° C with either 0.1% or 0.05% trypsin for 30 to 40 min, but complement components C3 and C4 were more sensitive to digestion. Although

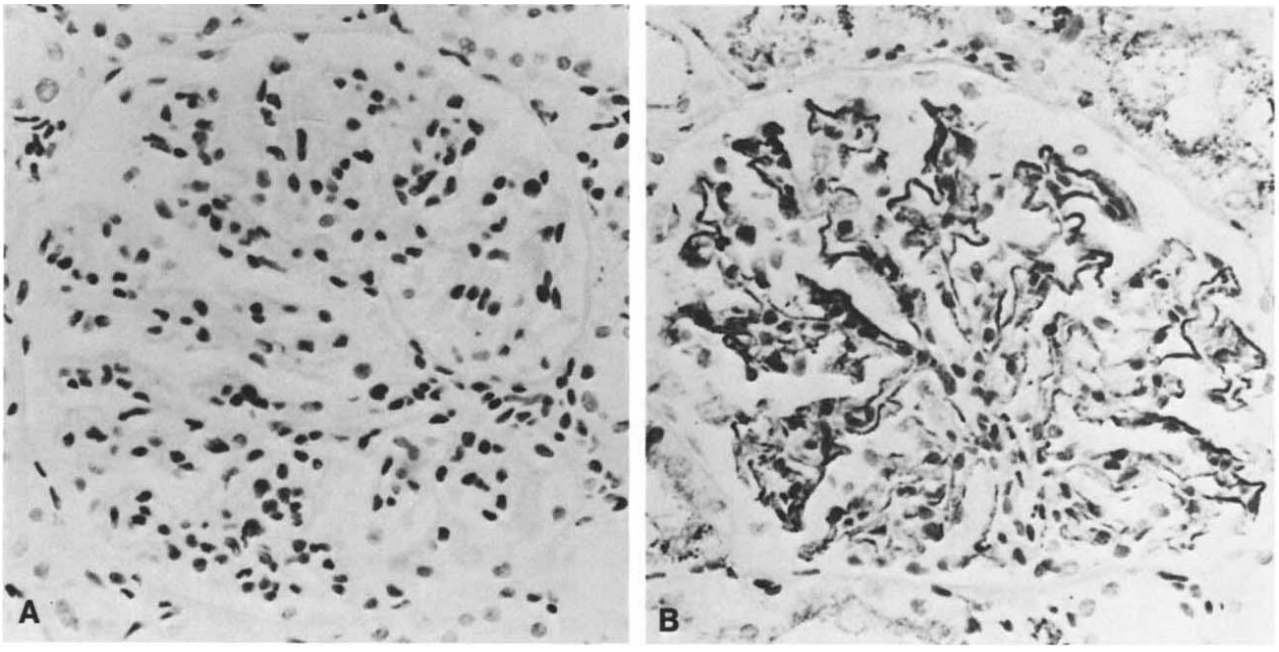

Fig. 1. *Membranoproliferative glomerulonephritis examined by immunoperoxidase (PAP) technique* **A** *without preincubation in trypsin and* **B** *after 40 min's incubation in 0.05% trypsin.* Complement, C3, granules are present after treatment with trypsin (hematoxylin counterstain; magnification, ×250).

they were present after 30 to 35 min's incubation in 0.1% trypsin solution, they became undetectable with longer digestion. With a weaker solution, 0.05% trypsin, they were clearly demonstrated following incubation for up to 50 min (Table 4). In all subsequent tests, therefore, 40 min's incubation in 0.05% trypsin was used. Longer treatments produced staining of collagen, digestion of nuclei and cytoplasm, and eventual disintegration of the section. There was clear separation of granular (Fig. 2) and linear (Fig. 3) patterns of deposition. Proteins in the glomerular capillary walls could be distinguished easily from those in the mesangium. The results obtained by the immunofluorescent and the trypsin-PAP methods are shown in Table 5. There is good correlation in most instances, and concordance is found in 81% of the observations. Immunoperoxidase methods were positive in 18 instances when immunofluorescence had been negative, and immunofluorescence was positive on 12 occasions when immunoperoxidase was negative. In all the discrepant results, only weak (+) or trace (±) reactions were recorded, indicating that the sensitivity of the two methods is similar.

The demonstration of protein in tissue sections is particularly useful in the study of renal disease.

Table 4. Trypsin incubation times and concentrations for the demonstration of different proteins by the immunoperoxidase (PAP) method[a]

Digestion time *min*	Demonstration of immunoglobulin heavy chains (IgG, IgM, IgA & IgD) and Clq		Demonstration of C4 and C3	
	0.1% Trypsin	0.05% Trypsin	0.1% Trypsin	0.05% Trypsin
20	–	–	–	–
25	–	–	–	–
30	+++	+++	++	+
35	+++	+++	++	++
40	++++	++++	–	+++
45	++++	++++	–	+++
50	++++	++++	–	+++
55	++++	++++	–	–
60	++++	++++	–	–

[a] Scoring is negative (–) to strong positive (++++).

Table 5. Correlation of results obtained by immunofluorescence (IF) method on frozen renal tissue and trypsin-immunoperoxidase (PAP) method on formalin-fixed tissue

	Total examined	PAP+ IF+	PAP– IF–	PAP+ IF–	PAP– IF+
IgG	28	19	6	1	2
IgA	24	8	12	3	1
IgM	21	10	5	5	1
IgD	21	2	17	—	2
C3	27	21	3	1	2
C4	21	2	12	5	2
Cl_q	19	2	12	3	2
Total		*64*	*67*	*18*	*12*

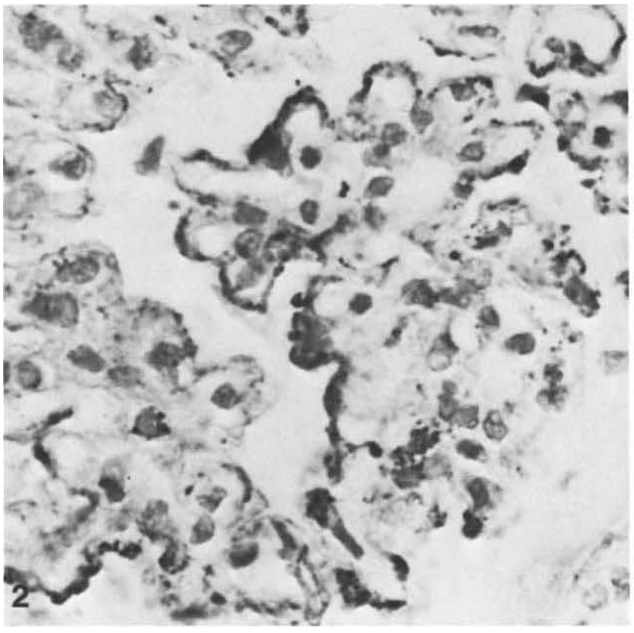

Fig. 2. *Granules of complement, C3 are seen within the basement membrane (hematoxylin counterstain; magnification, ×600).*

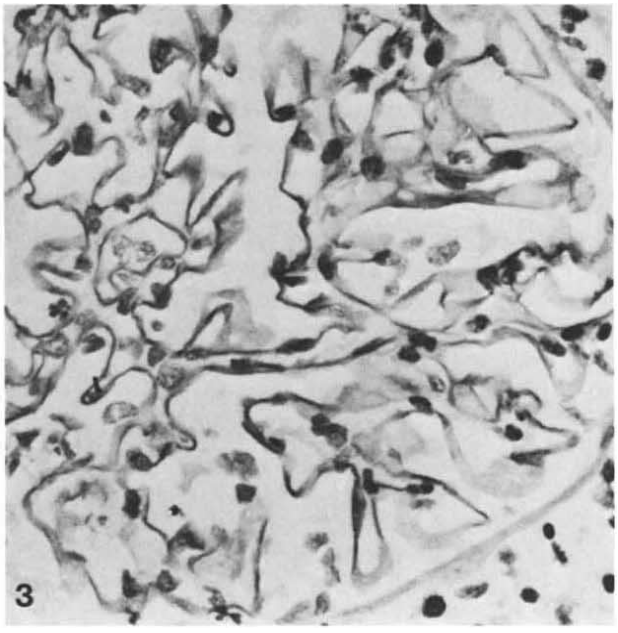

Fig. 3. *Linear deposit of IgG in glomerular basement membrane (hematoxylin counterstain; magnification, ×450).*

The advantages and disadvantages of immunoperoxidase methods compared to the immunofluorescent methods have been reviewed recently [12]. Until now, the demonstration of extracellular immunoglobulin with complement in formalin-fixed wax-embedded renal biopsy specimens has not been reported. After the tissue is carefully digested with trypsin, these proteins can be detected in routinely processed renal biopsy specimens that have been stored for a number of years. Thus, in biopsy specimens in which there is inadequate tissue for immunofluorescence or in highly infective material, serum protein deposits can be identified. Other protein antigens such as fibrinogen/fibrin and albumin can also be demonstrated in a similar manner (unpublished observations). Comparison of results from immunofluorescent and trypsin-PAP methods shows that the latter affords better localization of proteins within glomeruli. The method, however, is not always as sensitive. It is not clear why there is variation in detection of proteins by the two methods, although antisera from different commercial sources may have differing degrees of activity. With the immunofluorescent method, Huang, Minassian, and More [6] have detected several protein antigens (IgG, IgA, IgM, and fibrinogen/fibrin) in glomeruli after digestion with 0.1% trypsin solution for 30 min, 60 min, and 120 min to 8 hours. They reported, however, that the demonstration was unreliable and that complement could not be detected. The reason for their failure to detect complement is probably due to the duration of treatment and the concentration of trypsin that they used. As complement components C3 and C4 can be removed by overtreatment with 0.1% trypsin, it is necessary to examine the sections at intervals between 30 and 60 min if they are to be detected. Although it is known that trypsin can catalyze the hydrolysis of amino acids, particularly bonds of arginine and lysine [13], it is not clear whether this is important in the digestion of proteins which have been previously fixed in formalin. Curran and Gregory [7] suggest that trypsin incubation allows antisera to penetrate sections which are impermeable because of fixation. Although this may be partly responsible, the variable susceptibility of complement components indicates that this is not the only reason for the success of treatment with trypsin. Huang et al [6] have suggested that digestion alters the electrostatic charge of the tissue with resultant improvement in binding of antibody to antigen. They have also proposed that it may "unmask" immunoreactive sites on the protein, which seems likely.

In conclusion, trypsin digestion prior to the PAP technique can reveal glomerular, vascular, and tubular deposits of immunoglobulins IgG, IgA, IgM, and IgD and complement components C3, C4, and Clq in routinely fixed and processed needle-

biopsy specimens of the kidney. Solutions of 0.05% trypsin have been found to work most satisfactorily. With insufficient treatment, there is no immunohistochemical reaction, whereas excessive incubation produces loss of complement components C3 and C4, an increase in staining of connective tissue, and eventual disintegration of the section. The results of this method and those of conventional immunofluorescence performed on frozen renal tissue show good correlation. The use of the technique in retrospective studies has been demonstrated.

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